



Screening of native entomopathogenic nematodes against semi-looper (*Synegia* sp.) infesting black pepper (*Piper nigrum* L.)

Rashid Pervez*, S.J. Eapen¹ and Rajkumar²

Division of Nematology, ICAR-Indian Agricultural Research Institute, New Delhi-110 012, India

¹ICAR-Indian Institute of Spices Research, Kozhikode - 673 012, Kerala, India

²ICAR-Central Plantation Crops Research Institute, Kasaragod - 671 124, Kerala, India

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Abstract

Several insect pests damage and caused economic losses to black pepper (*Piper nigrum* L.). Among them semi-looper (*Synegia* sp.) is an important pest especially in younger vines of black pepper. Pesticides are currently being used to manage this pest leading to environmental and health concerns. Screening for virulence of eight native entomopathogenic nematodes were tested against the semi-looper (SL). The suitability of this insect for multiplying EPNs, attachment to and penetration into host was also studied. Among test EPNs, *Heterorhabditis* sp. (IISR-EPN 01), *Steinernema* sp. (IISR-EPN 02) and *O. gingeri* (IISR-EPN 07) were more virulent to semi-looper as they caused 100 per cent mortality to SL within 72 h. Maximum number of infective juveniles (IJs) attached into larva at 6 and 12 h post exposure. Maximum number of *Steinernema* sp. (IISR-EPN 02) IJs penetrated into larva (12.5 IJs per larva), followed by *Heterorhabditis* sp. (IISR-EPN 01) (8.7 IJs per larva) and only 5.9 IJs per larva of *O. gingeri* (IISR-EPN 07) penetrated into the test insect body. Maximum multiplication of IJs was observed with *O. gingeri* (IISR-EPN 07), which yielded 9,324 IJs per larva within 15 days, followed by *Oscheius* sp. (IISR-EPN 04) (8,638 IJs per larva) and *Oscheius* sp. (IISR-EPN 08) (8,236 IJs per larva). The infectivity of EPNs against semi-looper (*Synegia* sp.) is being reported for the first time which opens up a new vista in eco-friendly insect pest management in black pepper.

Keywords: Black pepper, entomopathogenic nematodes, semi-looper, *Synegia* sp.

Introduction

Black pepper production in Kerala is in steady decline due to several reasons, among which insect pests is a major constraints. Among them, semi-looper *Synegia* sp. (Lepidoptera: Geometridae) infestation causes reduction in pepper yield. The larvae of *Synegia* sp. feed on tender leaves and spikes of younger vines of black pepper during monsoon season (Devasahayam, 2008). The larvae are reported to damage up to 52 per cent of leaves and 17 per cent of spikes in Kozhikode district of Kerala (Premkumar and Devasahayam, 1989).

Pesticides are currently being used to manage this pest leading to environmental and health

concerns and result in the suppression of other naturally occurring biocontrol agents as well as developing resistance against pesticides. There is a need to identify suitable alternative methods for managing this insect pest. Recently, entomopathogenic nematodes (EPNs) are emerging as potent biocontrol agents against a variety of insect pests of crops due to their wide host range, easy to handle, short life cycle, environmentally safe and economically multiplied at large scale (Ali *et al.*, 2005; 2008; Pervez *et al.*, 2007; Pervez *et al.*, 2012). EPNs are symbiotically associated with bacteria which are released into the insect hemocoel, causing causing septicemia and death of the insect (Pervez *et al.*, 2015a).

*Corresponding Author: rashidpervez2003@gmail.com

Some EPNs are more virulent against a particular insect group than against another insect group. In addition, proper combination of the EPN to the host entails virulence, host finding and ecological factors. If EPN does not possess a high level of virulence towards the target pest, there is little hope of success (Georgis, 2004). Bedding *et al.* (1983) indicated the importance of screening of several EPNs against the target insect in the laboratory before commencing field evaluations.

EPNs have been found effective against a variety of insect pest infesting spice crops (Pervez *et al.*, 2012; 2014 a,b; 2016). There is scope of using EPN in black pepper, which will take care of insect pest and reduce the damage caused by them and thus these will be an ideal alternative to chemicals, economical and will fit in long term pest management system without risk to non-target organisms.

Objective of the study was to screen the virulence of native EPNs against semi-looper (*Synegia* sp.) infesting black pepper. Attachment, penetration and suitability of this insect for multiplication of infective juveniles (IJs) of EPNs, LC_{50} and LT_{50} of the promising EPNs were also calculated.

Materials and methods

Sources of entomopathogenic nematode

The IJs of eight native EPNs were obtained from nucleus culture of nematodes maintained in the Nematology Laboratory, ICAR-Indian Institute of Spices Research (IISR), Kozhikode (Table 1). All tested EPNs were cultured on greater wax moth, *Galleria mellonella* larvae. The IJs were surface sterilized in 0.1 per cent hyamine solution and stored in distilled water in tissue culture flasks for study.

Insect sources

Greater wax moth, *G. mellonella* larvae cultured as per the procedure described by Kaya and Stock (1997). The test insect, semi-looper (*Synegia* sp.) was collected from ICAR-IISR Experimental Farm, Peruvannamuzhi, Kozhikode and ICAR-CPCRI Experimental Farm, Kasaragod. Larvae of same size were taken for present study.

Infectivity of EPNs against semi-looper

Infectivity of EPNs against semi-looper (*Synegia* sp.) was tested in Petri plates. For this, single insect was kept in each plate with small piece of black pepper leave and 100 IJs in 0.5 mL water of each tested species of EPNs (Table 1) were inoculated and their mortality was recorded at 24 h interval. Each species of EPN was tested separately and singly. The experiment was conducted at room temperature and replicated six times along with control. The mortality was calculated according to following formula.

$$\text{Mortality (\%)} = D/N \times 100$$

where, D = number of dead larvae and

N = total number of larvae

Determination of lethal concentrations (LC_{50})

Lethal concentrations were calculated using four densities viz., 25, 50, 75 and 100 IJs of three promising EPNs, *Heterorhabditis* sp. (IISR-EPN 01), *Steinernema* sp. (IISR-EPN 02) and *O. gingeri* (IISR-EPN 07) against semi-looper (*Synegia* sp.). Five insect were kept per plate with small piece of black pepper leaves and suspension of each density of tested EPN was inoculated into Petri plates lined with filter paper at the bottom of the plate. Mortality of semi-looper was recorded at 72 h. The experiment was conducted at room temperature and replicated six times along with controls. The percentage mortality calculated using Abbott's formula (Abbott, 1925) and mean values were worked out. The LC_{50} values were calculated using regression analysis. Correlation between concentrations and mortality of insect was also determined.

Determination of lethal time (LT_{50})

Lethal time was calculated using one EPN density @ 100 IJs per Petri plate. All experimental condition same as to determine LC_{50} except the mortality of the larvae recorded at 24, 48, 72 and 96 h of post inoculation. The percentage mortality calculated using Abbott's formula (Abbott, 1925) and mean values were worked out. The LT_{50} values were calculated using regression analysis. Correlation between exposure time and mortality of semi-looper (*Synegia* sp.) was also calculated.

Attachment of IJs to semi-looper

Attachment of three promising EPNs viz., *Heterorhabditis* sp. (IISR-EPN 01), *Steinernema* sp.

Table 1. Entomopathogenic nematodes used in bioassay

Family	Genus	Isolates
Heterorhabditidae (Poinar, 1976)	<i>Heterorhabditis</i> sp. (Poinar, 1976)	<i>Heterorhabditis</i> sp. (IISR-EPN 01) (Pervez <i>et al.</i> , 2014a)
Steinernematidae (Chitwood and Chitwood, 1937)	<i>Steinernema</i> spp. (Steiner, 1923)	<i>Steinernema</i> sp. (IISR-EPN 02) (Pervez <i>et al.</i> , 2014a) <i>S. ramanai</i> (IISR-EPN 03) (Pervez <i>et al.</i> , 2011) <i>S. carpocapsae</i> (IISR-EPN 06) (Pervez <i>et al.</i> , 2015b)
Rhabditidae (Poinar, 1976)	<i>Oscheius</i> spp. (Andrassy, 1976)	<i>Oscheius</i> sp. (IISR-EPN 04) (Pervez <i>et al.</i> , 2014a) <i>Oscheius</i> sp. (IISR-EPN 05) (Pervez <i>et al.</i> , 2014b) <i>O. gingeri</i> (IISR-EPN 07) (Pervez <i>et al.</i> , 2013) <i>Oscheius</i> sp. (IISR-EPN 08) (Pervez <i>et al.</i> , 2014b)

(IISR-EPN 02) and *O. gingeri* (IISR-EPN 07) to semi-looper (*Synegia* sp.) tested in six well plates. One larva of semi-looper was kept in each well and 100 IJs of tested EPNs were released and kept at room temperature. The treatments were replicated six times. The number of IJs attached to the semi-looper was counted by the washing the insect in a counting dish with distilled water after 3, 6, 12 and 24 h.

Penetration of IJs in to semi-looper

The penetration rate assay was conducted as procedure described by Pervez *et al.* (2014b). About 100 IJs of promising EPNs viz., *Heterorhabditis* sp. (IISR-EPN 01), *Steinernema* sp. (IISR-EPN 02) and *O. gingeri* (IISR-EPN 07) were inoculated in the well plate containing one larva per well and kept at room temperature. Each treatment replicated six times. Number of penetrated IJs was determined by dissecting the dead cadaver after 72 h.

Multiplication of EPNs

EPN infected dead larva were removed from the Petri plate and kept on the White trap (White, 1927) for emergence of IJs from the body of larva. IJs were collected daily, till the emergence stopped in about 15 days. Collected IJs were counted three times under a stereoscopic binocular microscope with the help of Syracuse counting dish and mean values were worked out.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) and means compared according to Duncan's multiple range tests. Before analysis, data

of mortality and multiplication of the EPNs were square root transformed and those of percentages of insect mortalities were arcsine transformed. All means were transformed back to the original units for presentation.

Results and discussion

The results indicated that, all tested EPNs were virulent against semi-looper (*Synegia* sp.). Although, attachment, penetration, mortality (%) and multiplication number of IJs varied from EPN to EPN. However, concentrations and exposures time had a significant effect on the mortality of semi-looper.

Infectivity of EPNs against semi-looper

All test EPNs were virulent against semi-looper, but insect mortality time was varied. Among test EPNs, *Heterorhabditis* sp. (IISR-EPN 01), *Steinernema* sp. (IISR-EPN 02) and *O. gingeri* (IISR-EPN 07) were more virulent to semi-looper as they caused 100 per cent mortality to the SL within 72 h, followed by *S. ramanai* (IISR-EPN 03), *S. carpocapsae* (IISR-EPN 06) and *Oscheius* sp. (IISR-EPN 08). The other species took 120 h, to kill the test insect. No mortality was observed in control (Fig. 1).

Some EPN species or strains were reported as highly host specific and demonstrate great variation in their infectivity considerably in different hosts and none of the species or strains of EPN infected all the insect pests (Shapiro and Mc Coy, 2000; Ali *et al.*, 2008). Gaugler *et al.* (1989) found a similar variation among geographically distant strains of

S. carpocapsae. Our studies supported by earlier workers, the variation in mortality percentage within Steinernematids, Heterorhabditids and Rhabditid groups indicated that, neither group was superior to the other. The variation in efficiency of the various EPNs may be due to the difference in the bacterial symbionts (Boemare, 2002) and preferential feeding behaviour (Bilgrami and Gaugler, 2009; Pervez, 2011). Hence, laboratory screening of EPNs for infectivity an important component of developing a biological control programme for a particular pest (Ricci *et al.*, 1996).

Determination of LC_{50}

Result shows that, mortality per cent of semi-looper increases with the increase the number of IJs. Among the test densities, maximum mortality was found in 100 IJs per plate, whereas minimum mortality was in 25 IJs per plate. Mortality was also observed when treated @ 25 IJs per plate for all test EPNs except *O. gingeri* (IISR-EPN 07), whereas it was observed at 50 IJs per plate (Fig. 3).

A significant and positive correlation was found between IJs density and insect mortality. Probit analysis indicated that, *Steinernema* sp. (IISR-EPN 02), and *Heterorhabditis* sp. (IISR-EPN 01) required less number (51 and 54 IJs per larva, respectively), whereas *O. gingeri* (IISR-EPN 07) need maximum number (78 IJs per larva) to provoke 50 per cent mortality of semi-looper at 72 h post inoculation (Fig. 2).

Determination of LT_{50}

Semi-looper mortality per cent increases with the increase the exposure time (Fig. 4). All tested EPNs induced mortality of semi-looper within 24 h. A significant and positive correlation was found between exposure time and mortality. Time assay response revealed that *Steinernema* sp. (IISR-EPN 02) and *O. gingeri* (IISR-EPN 07) took less time (31 and 38 h, respectively) to kill 50 per cent semi-looper, whereas *Heterorhabditis* sp. (IISR-EPN 01) took more time to kill insect (42 h) (Fig. 3).

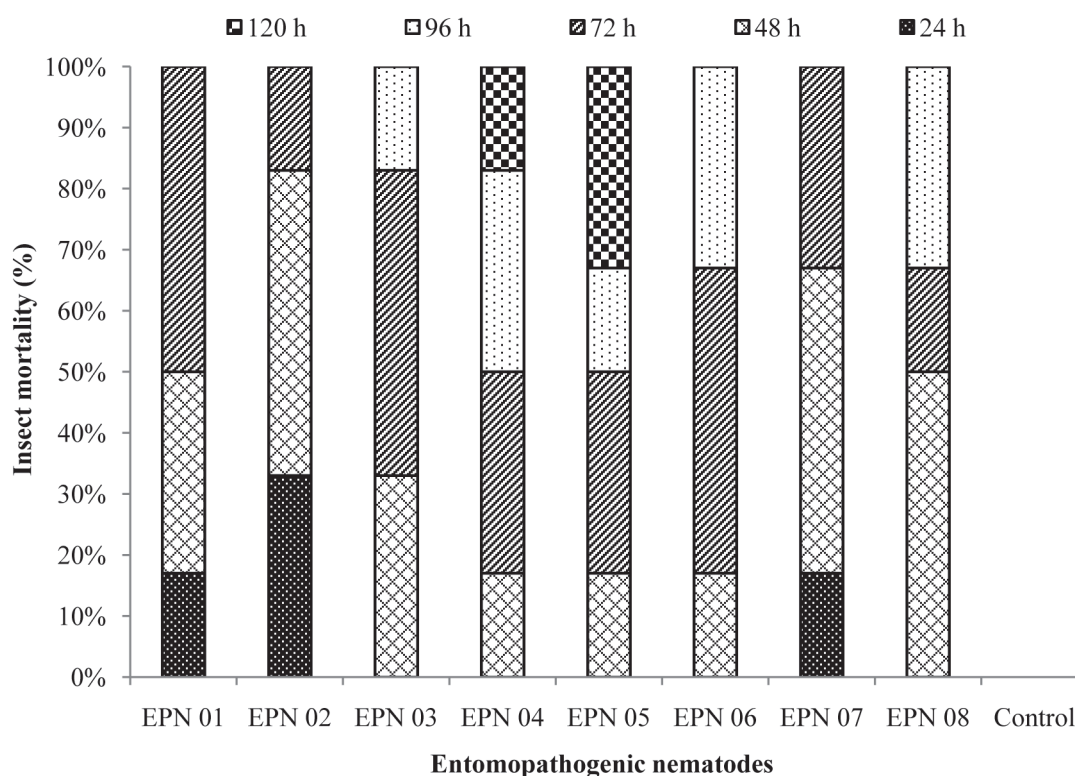


Fig. 1. Mortality of semi-looper *Synegia* sp. EPN 01 - *Heterorhabditis* sp.; EPN 02 - *Steinernema* sp.; EPN 03 - *S. ramanai*; EPN 04 - *Oscheius* sp.; EPN 05 - *Oscheius* sp.; EPN 06 - *S. carpocapsae*; EPN 07 - *O. gingeri* and EPN 08 - *Oscheius* sp.

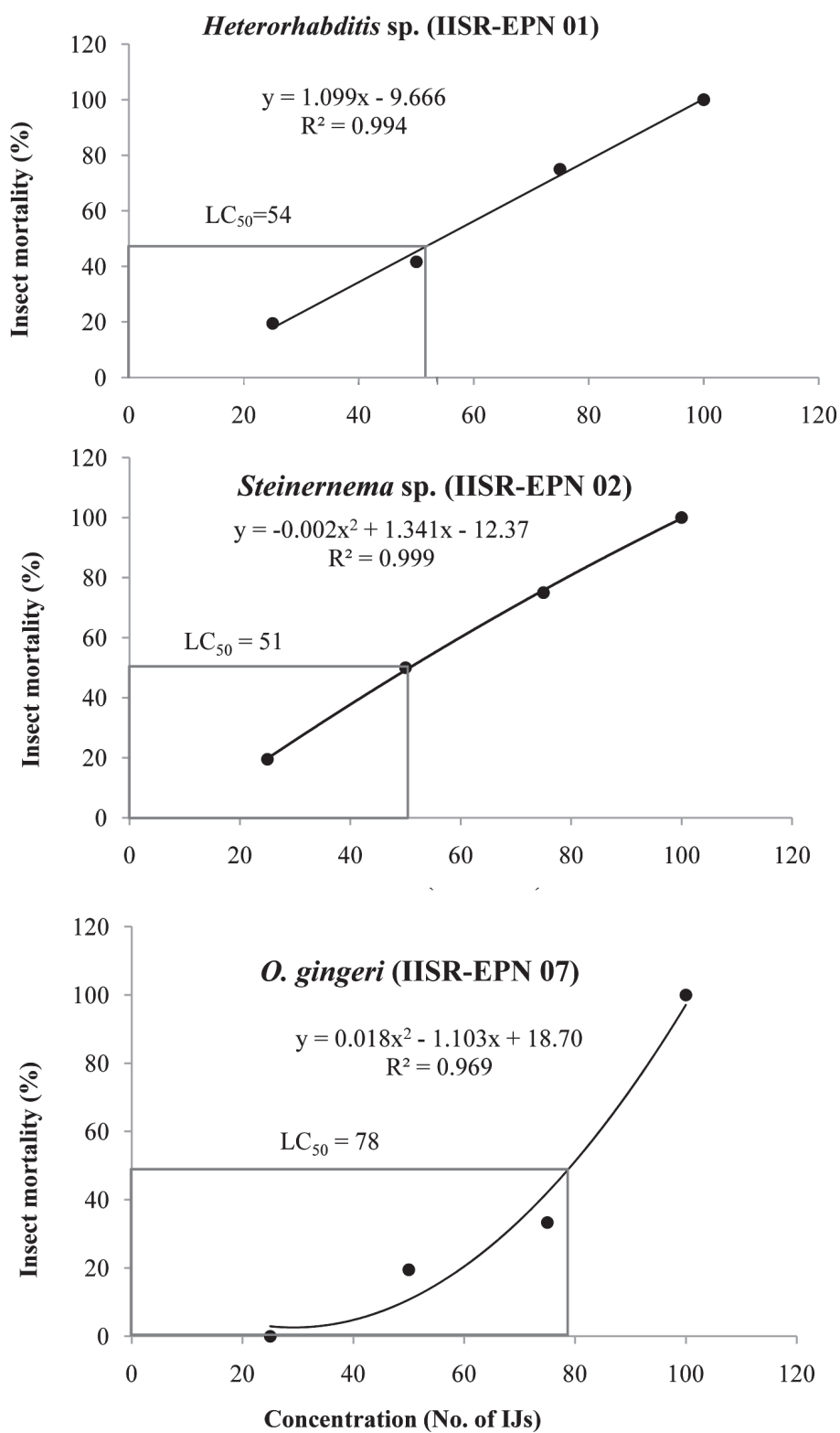


Fig. 2. LC_{50} value of the entomopathogenic nematodes against semi-looper *Synegia* sp.

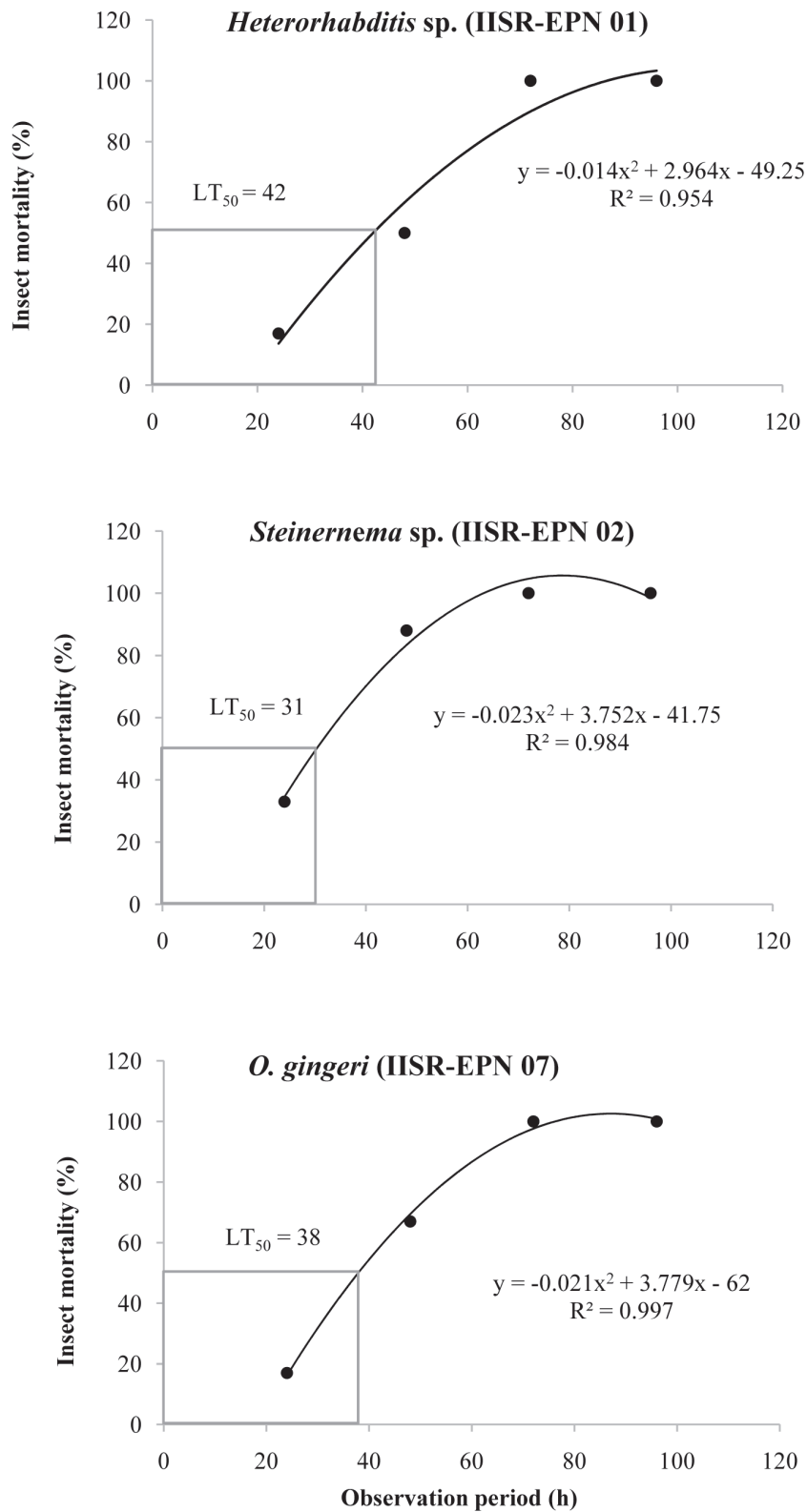


Fig. 3. LT₅₀ value of the entomopathogenic nematodes against semi-looper *Synegia* sp.

Although, the semi-looper was susceptible to test EPNs, there were differences among these EPNs in their ability to mortality of the insect. Among test EPNs, *Steinernema* sp. (IISR-EPN 02) was most virulent against semi-looper. The mortalities were higher, LC_{50} values were lower and LT_{50} values were shorter for this EPN. These differences may be due to difference of the origins of the strains (Mbata and Sapiro-Ilan, 2005; Canhilal, 2012) and preferential feeding behaviour (Bilgrami and Gaugler, 2009; Pervez, 2011).

Attachment of IJs to semi-looper

All tested EPNs IJs attached to SL, whereas significant differences ($df = 7, 41$; $F = 5.69$; $p = 0.003$) was recorded in the attachment of IJs. Among the test EPNs, the maximum number of IJs attached to larva at 6 and 12 h post exposure (Fig. 4).

Penetration of IJs into semi-looper

All tested EPNs IJs penetrated into semi-looper, whereas significant differences ($df = 6, 38$; $F = 6.12$; $p = 0.003$) was found in the penetration of IJs. Among the tested species, the maximum number of *Steinernema* sp. (IISR-EPN 02) IJs penetrated into larva (12.5 IJs per larva), followed by *Heterorhabditis* sp. (IISR-EPN 01) (8.7 IJs per

Table 2. Number of IJs penetrated in to semi-looper *Synegia* sp.

Entomopathogenic Nematodes	Number of IJs per larva
<i>Heterorhabditis</i> sp. (IISR-EPN 01)	8.7 ^{ab}
<i>Steinernema</i> sp. (IISR-EPN 02)	12.5 ^a
<i>Oscheius gingeri</i> (IISR-EPN 07)	5.9 ^c

Values followed by different letters are significantly different

larva). However, the minimum number (5.9 IJs per larva) of *O. gingeri* (IISR-EPN 07) IJs penetrated into the test insect body (Table 2).

Penetration of the IJs of *Steinernema* spp. and *Heterorhabditids* sp. showed highest penetration and was superior to *Oscheius* sp. The present findings supported by previous study (Sankaranarayanan *et al.*, 2011; Pervez *et al.*, 2011; 2014a,b). The differences in penetration of IJs in the present study might be due to the infection strategies of EPNs. The rate of penetration could be used as a real measure of host infection. These results might suggest that different species of EPN or natural variability within semi-looper. However, Tomalak (2004) observed variation in IJs penetration between native EPNs originating from collection sites located within a short distance, suggesting that variation in infection can be

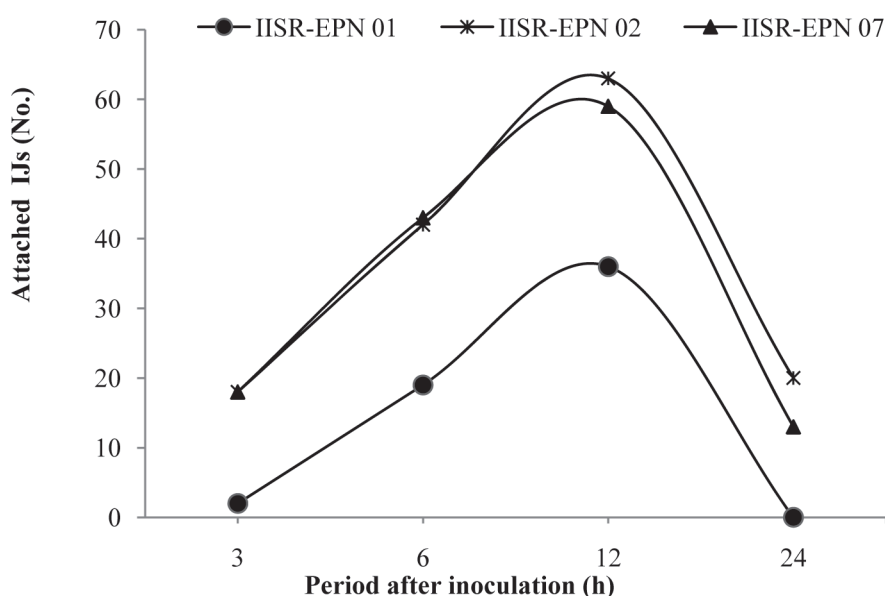


Fig. 4. Entomopathogenic nematodes attached to semi-looper *Synegia* sp. IISR-EPN 01- *Heterorhabditis* sp. (IISR- EPN 01); IISR-EPN 02 - *Steinernema* sp. (IISR-EPN 02) and IISR-EPN 07 – *O. gingeri*

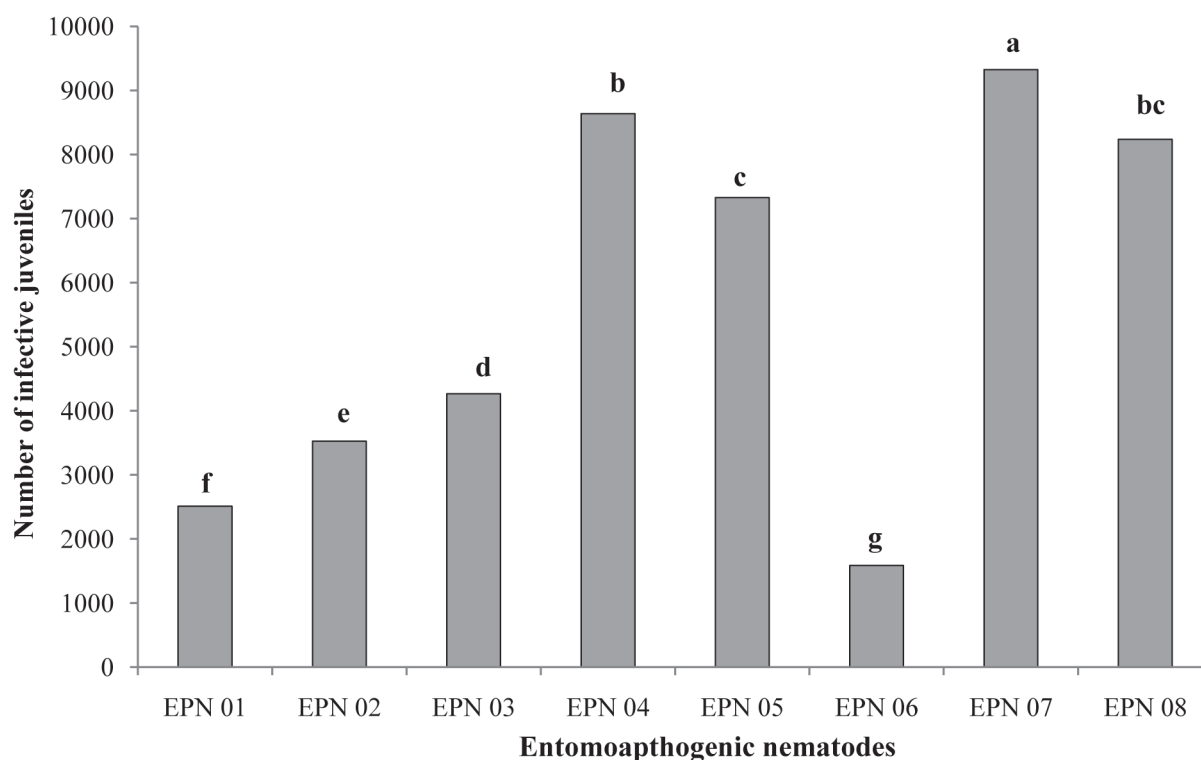


Fig. 5. Multiplication of infective juveniles (IJs) of entomopathogenic nematodes. EPN 01- *Heterorhabditis* sp. (IISR- EPN 01); EPN 02 - *Steinernema* sp. (IISR-EPN 02); EPN 03 - *S. ramanai*; EPN 04 - *Oscheius* sp. (IISR-EPN 04); EPN 05 - *Oscheius* sp. (IISR-EPN 05); EPN 06 - *S. carpocapsae*; EPN 07 – *O. gingeri* and EPN 08 - *Oscheius* sp. (IISR-EPN 08). Bars with different letters are significantly different

observed between EPNs of different species but also between isolate of same EPN species (Mwaitulo *et al.*, 2011; Pervez *et al.*, 2014).

Multiplication of EPNs

All tested EPNs were multiplied on the semi-looper but rate of multiplication of IJs varied. Among the tested EPNs, maximum multiplication of IJs was observed with *O. gingeri*, which yielded 9,324 IJs per larva, followed by *Oscheius* sp. (IISR-EPN 04) (8,638 IJs per larva) and *Oscheius* sp. (IISR-EPN 08) (8,236 IJs per larva). However, *S. carpocapsae* (IISR-EPN 06) yielded the least number of IJs (1,586 IJs per larva) (Fig. 5).

Mass produced of EPNs *in vivo* where the insect serves as a small biological reactor. Greater wax moth, *G. mellonella* has been widely used for *in vivo* mass production of EPNs (Ali *et al.*, 2005; 2008). Other insects, such as spotted borer, *Chilo sacchariphagus indicus* (Karunakar *et al.*, 1992; 1999), gram pod borer, *Helicoverpa armigera* and rice moth, *Corcyra cephalonica* (Ali *et al.*, 2008;

Pervez, 2010), mustard saw fly, *Athalia proxima* (Pervez *et al.*, 2007), green bug, *Nezara viridula* (Pervez *et al.*, 2008), tobacco caterpillar, *Spodoptera litura* (Pervez and Ali, 2009), legume pod borer, *Maruca vitrata* (Pervez and Ali, 2011), shoot borer, *Conogethes punctiferalis* and hairy caterpillar, *Euproctis* sp. (Pervez *et al.*, 2012) and turmeric leaf feeder, *Lema* sp. (Pervez *et al.*, 2014) also suitable for multiplication of various species of *Steinernema*, *Heterorhabditis* and *Oscheius* with varying yields of IJs depending upon the size of larvae of the test insects and reproductive behaviour of the EPN species (Pervez and Ali, 2013; Pervez, 2010; 2011).

Conclusion

The infectivity of EPNs against semi-looper (*Synegia* sp.) is reported for the first time which will be valuable in designing eco-friendly insect pest management in black pepper. Among the EPNs studied, *Steinernema* sp. (IISR-EPN 02) was more virulent against semi-looper (*Synegia* sp.) infesting black pepper. Further work is required to confirm

these results in the green house and field conditions with this promising EPN against semi-looper (*Synegia* sp.).

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